Research Article

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Assessment of LXRα agonist activity and selective antiproliferative efficacy: a study on different parts of *Digitalis* species

LXRα agonist ve seçici antiproliferatif etkinin değerlendirilmesi: *Digitalis* türlerinin farklı kısımları üzerinde bir çalışma

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Abstract

Objective: Aerial parts and roots of three *Digitalis* species (*Digitalis davisiana* Heywood, *Digitalis viridiflora* Lindley and *Digitalis grandiflora* Miller; Plantaginaceae) were investigated from the viewpoint of cytotoxicity to identify the biologically active parts. Playing a role in cell proliferation and tumor growth, LXRα agonist activity also has become of interest to researchers investigating its relationship with the cytotoxicity.

Materials and methods: Cytotoxicity of aqueous extracts was determined through HEp-2, HepG2 and 3Y1 cells using MTT method. LXRα agonist activity was determined through luciferase reporter gene assay on HEK293 cells.

Results: Tested extracts showed strong cytotoxicity on HEp-2 cells with IC_{50} values between 19.7 and 79.6 µg/mL. Cytotoxicity on HepG2 cells was found to be lower (IC_{50}; 211.4–2152.9 µg/mL). On 3Y1 cells, extracts showed concentration dependent cytostatic activity (IC_{50}; 87.9–772.3 µg/mL). Extracts showed cytotoxicity on HEK293 cells at a concentration of 100 µg/mL; therefore, dilutions were made. However, due to dilutions, LXRα agonist activity was found to be low.

Conclusion: The extracts exhibited selective cytotoxicity on cancer and non-cancerous cells. Moreover, the selectivity was seen between different cancer cells. Any relationship could not be found between cytotoxicity and LXRα agonist activity, due to the low agonist effects. Further investigations are needed to clarify the mechanism of activity.

Keywords: *Digitalis* species; Plantaginaceae; Cytotoxicity; HEp-2 cell line; HepG2 cell line; Liver X receptor.

Öz

Amaç: Üç *Digitalis* türü; *D. davisiana* Heywood, *D. viridiflora* Lindley ve *D. grandiflora* Miller (Plantaginaceae)'in toprak üstü kısımları ve kökleri, biyolojik olarak aktif kısımları tanımlamak için sitotoksisite açısından incelenmiştir. Hücre çoğalması ve tümör büyümesindeki rolü nedeniyle, LXRα agonist etki de, sitotoksisite ile ilişkisinin belirlenmesi bakımından, bu çalışmada bir ilgi odağı olmuştur.

Gereç ve Yöntem: Sulu ekstrelerin sitotoksik etki çalışmaları HEp-2, HepG2 ve 3Y1 hücreleri üzerinde MTT yöntemi kullanılarak gerçekleştirilmiştir. LXRα agonist etkiyi belirlemek için, HEK293 hücreleri kullanılarak lusiferaz bildirici gen testi uygulanmıştır.

Bulgular: Çalışılan ekstreler, HEp-2 hücreleri üzerinde 19.7–79.6 µg/mL IC_{50} değerleri ile güçlü bir sitotoksisite göstermiştir. HepG2 hücreleri üzerinde sitotoksisite daha düşük bulunmuştur (IC_{50}; 211.4–2152.9 µg/mL). 3Y1 hücrelerinde, ekstreler konsantrasyona bağlı sitostatik etki göstermiştir (IC_{50}; 87.9–772.3 µg/mL). Ekstreler, 100 µg/mL

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konsantrasyonda HEK293 hücreleri üzerinde sitotoksisite göstermiş, bu yüzden seyreltmeler yapılmıştır. Ancak seyreltmeler nedeniyle LXRα agonist etkilerinin düşük olduğu bulunmuştur.

Sonuç: Ekstreler, kanserli ve normal hücrelerde seçici sitotoksisiteye sahiptir. Ayrıca, seçicilik, farklı kanser hücreleri arasında da görülmüştür. Ekstrelerin düşük agonist etkilerinden dolayı sitotoksisite ve LXRα agonist etki arasında herhangi bir ilişki bulunamamıştır. Etki mekanizmasını açıklığa kavuşturmak için daha fazla araştırmaya ihtiyaç vardır.

Anahtar kelimeler: *Digitalis* türleri; Plantaginaceae; Sitotoksisite; HEp-2 hücre serisi; HepG2 hücre serisi; Liver X reseptör.

Introduction

Digitalis species have been investigated in detail especially for cardioactive glycosides. Due to their positive inotropic effect, cardioactive glycosides have been used in the treatment of congestive heart failure for a long while [1]. *Digitalis* species are the most known among plants which contain cardioactive glycosides. Especially, *Digitalis lanata* and *Digitalis purpurea* are highly significant for their cardioactive glycosides [2].

Phytochemical studies on *Digitalis* genus have already identified other biologically active compounds, such as flavonoids, anthraquinones, phenylethanoid glycosides and other similar phenolic compounds, which are especially rich in phenylethanoid glycosides [3, 4]. In the Flora of Turkey, *Digitalis* genus are represented by nine species, but there is no evidence that it was used as a traditional medicine in Anatolia [5].

In some previous reports cytotoxicity has been reported in *Digitalis* species against different cells [6, 7]. *Digitalis* species also have emetic, antibacterial, antiviral, antifungal, antiinflammatory and antioxidant activities [8–11].

Recent studies highlighted the role of Liver X receptors (LXR) in cancer, and LXR activation seems to be protective in cancer prevention [12, 13]. Consequently, in cancer therapy, LXR has become a target recently. LXR is a member of nuclear receptor superfamily. Two LXRs, LXRα and LXRβ, have been expressed in different tissues. While LXRα is expressed highly in liver, intestine, kidney, spleen, macrophages and adipose tissue, LXRβ is expressed more ubiquitously [14]. Nuclear receptors are responsible for the regulation of gene transcription and intracellular function. LXR is critical in lipid metabolism as well as playing a role in de novo synthesis of cholesterol, excretion and detoxification of bile acids, or lipids, glucose homeostasis and neurological functions, and also have a crucial role in inflammatory responses [15, 16]. Recent studies show that LXR agonists might have a protective role for various cancers. For instance, it was shown that they decreased the risk for breast, gastric, prostate and lung cancers [15]. LXR agonists also inhibits cell proliferation in different cell lines, such as the prostate, breasts, liver, lungs, cervical, epidermis, skin melanoma, and bone to squamous carcinomas [17].

The aim of this study is to identify the biologically active parts of the genus plants from the view point of cytotoxicity. Playing a role in cell proliferation and tumor growth, LXRα agonist activity has also become the key interest in this study in order to identify the relation with the cytotoxicity.

We sought to examine the cytotoxic activity of the aerial parts and the roots obtained from three different *Digitalis* species (*Digitalis davisiana*, *Digitalis viridiflora* and *Digitalis grandiflora*) against two different cancer cells; HEp-2 (Human larynx epidermoid carcinoma) and HepG2 (Human hepatocelular carcinoma) with 3Y1 (rat fibroblast cell) non-cancerous cell using MTT method. 3Y1 non-cancerous cell line was used for the determination of selectivity on cancer cells. LXRE reporter gene assay was used to determine LXRα agonist activity on HEK293 cell line.

Materials and methods

Cell lines and cell culture media

HEp-2 (Human larynx epidermoid carcinoma) and HEK293 (Human embryonic kidney) cell lines were obtained from Riken Bioresource Center Cell Bank (Ibaraki, Japan). HepG2 (Human hepatocelular carcinoma) cell line was obtained from JCRB Cell Bank (Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan). 3Y1 (rat fibroblast cell) cells were provided by the Health Science Research Resources Bank (Osaka, Japan). Minimum Essential Medium Earle's salts (MEM's Earle), Dulbecco's Modified Eagle's Medium, tyripsin, sodium dodecyl sulfate and thiazolyl blue tetrazolium bromide (MTT) were obtained from Sigma Aldrich (St. Louis, MO, USA). Fetal bovine serum was purchased from Biowest. Penicillin, streptomycin, MEM non-essential amino acids solution were obtained from Wako Pure Chemical Industries (Osaka, Japan). T0901317 was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA).

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Plant material

Voucher specimens for all plants were deposited in Hacettepe University Faculty of Pharmacy Herbarium (HUEF). *Digitalis davisiana* Heywood was collected from Antalya, Turkey (HUEF11004) in June, 2011. *Digitalis grandiflora* Miller (HUEF13007) and *D. viridiflora* Lindley (HUEF13008) were collected from Kırklareli, Turkey in July, 2013. *Digitalis davisiana* was identified by Prof. Dr. Hayri Duman (Gazi University, Ankara, Turkey), *D. grandiflora* and *D. viridiflora* were identified by Dr. Z. Ceren Arıtuluk (Hacettepe University, Ankara, Turkey).

Extraction

The same extraction method was used for all plants. Plants were dried in appropriate conditions, aerial parts (AP) and the roots (R) were seperated, powdered and 10 g of both parts were extracted with MeOH three times for 6 h. Methanol was purchased from Sigma Aldrich (St. Louis, MO, USA). Extracts were dried by evaporation under a vacuum at 40°C. Then, they were dissolved in water and partitioned with petroleum ether to discard lipophylic fractions. Petroleum ether was purchased from Merck (Darmstadt, Germany). Aquoeus extracts of both aerial parts (APE) and roots (RE), were used for cytotoxicity tests. The following are the yields of aquoeus extracts of the dried plants: *D. davisiana* APE, 12%; *D. davisiana* RE, 14%; *D. grandiflora* APE, 9%; *D. grandiflora* RE, 12%; *D. viridiflora* APE, 14%; *D. viridiflora* RE, 11%.

Cytotoxic activity

HEp-2, HepG2 and 3Y1 cells were used for cytotoxic activity tests. Cells were seeded in a 96-well plate at a density of 1×10^5 cells/mL for HEp-2 cells, 4×10^5 cells/mL for HepG2 cells and 5×10^4 cells/mL for 3Y1 cells. MEM's Earle medium for HEp-2 and 3Y1 cells and DMEM for HepG2 cells were used. Cells were cultured in respective culture media supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 µg/mL streptomycin in a humidifier with 5% CO_2 in the air at 37°C for 24 h. The cells were then treated with different concentrations of samples for the next 48 h. After incubation, the cells were washed and replaced with a fresh medium. Ten microliter of MTT solution (5 mg/mL in PBS) was added and incubated for 4 h. After that, 100 µL of 10% SDS (Sodium dodecyl sulfate) was added to each well to dissolve formazan crystals. The absorbance was measured at 570/620 nm using microplate reader. Results were given as a percentage of inhibitory effect on treated cells to untreated cells that served as the control [18]. Three independent test results were considered, averages and standard deviations were calculated by GraphPad Prism 7 software (San Diego, CA, USA) and shown in the figures (Figures 2–4).

LXRE reporter gene assay

HEK293 cells were cultured in a minimum essential medium supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 50 U/mL penicillin, and 50 µg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Calcium phosphate co-precipitation method was used for transfection. For LXR luciferase reporter assay, pBApo-CMW-hLXR-α (30 ng), pGL4.1-DR4-Luc (120 ng), pCMX-β-gal expression vector (30 ng) and carrier DNA pUC18 were used to yield a total of 600 ng of DNA per well. After 6 h, cells were treated with test samples for 36 h incubation. The luciferase and β-galactosidase activities were analyzed from cell lysates using a luminescence reader and a spectrophotometer, respectively. The results were given as fold induction values relative to vehicle treated cells after normalization of luciferase activity by β-galactosidase activity [19]. A LXRα agonist T0109317 was used as positive control and vehicle treated cells were used as negative control in the assay. Three independent test results were considered, averages and standard error means were calculated and shown in the figure (Figure 1).

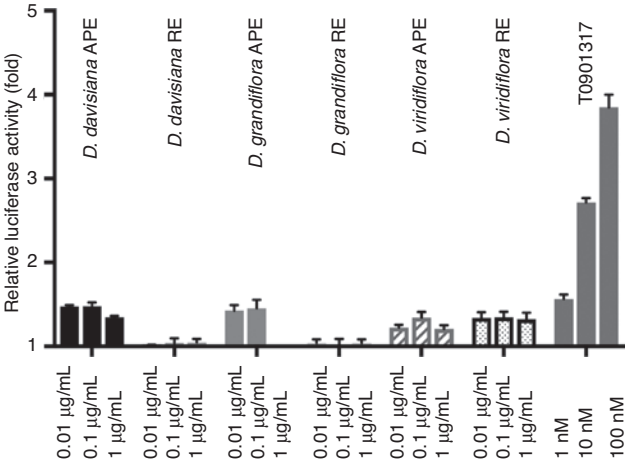


Figure 1: Screening of aqueous extracts obtained from the aerial parts and roots of *D. davisiana*, *D. grandiflora* and *D. viridiflora* for Liver X receptor (LXR) agonist activity using LXRE reporter gene assay.
Three independent test results were considered, averages and standard error means were calculated and shown in the figure.

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Results

This is a comparative study on three different *Digitalis* species collected from different regions of Turkey – each species having different variations in chemical composition between the aerial parts and the roots of each plant. The aim of the study was to identify cytotoxic activity against different cancer cells and determine the biologically active part of the selected plants. As LXR plays a role in cell proliferation and tumor growth, LXRα agonist activity was used to search for its relation with the cytotoxicity.

This research is the first investigation on *Digitalis* species for LXRα activity with luciferase reporter assay. According to the protocol of the laboratory, at first 100 μg/mL of concentration were used in assay. However, the results showed that *Digitalis* extracts have cytotoxic activity on HEK293 cell line in this concentration; and therefore dilutions were prepared in the following experiments. Extracts were tested at the concentration range of 0.01, 0.1 and 1 μg/mL. T09011317 was used as positive control at the concentrations of 1, 10 and 100 nM. The results were given as fold induction values normalized by β-galactosidase. After dilutions, LXRα activity was again tested; however due to the dilutions, activity was found to be low (Figure 1). The reason for the low activity could be either low concentration or no potential effect on LXRα receptors.

The cytotoxic activity results indicated that all six extracts showed similarly strong activity against HEp-2 cells with IC_{50} values in a range of 19.7–79.6 μg/mL, in a concentration dependent manner (Figure 2, Table 1), although the roots were found to be more active than the aerial parts against HEp-2 cells (Figure 2). *Digitalis davisiana* RE showed the highest cytotoxicity with a IC_{50} value of 19.7 μg/mL. At 400 μg/mL concentration, *D. grandiflora* RE and *D. viridiflora* RE also showed high cytotoxicity against HEp-2 cell line: cell viability was about 10% for both extracts. The IC_{50} values of the extracts are given in Table 1. The high cytotoxic activity of these extracts suggests their high potential for positive results in future studies.

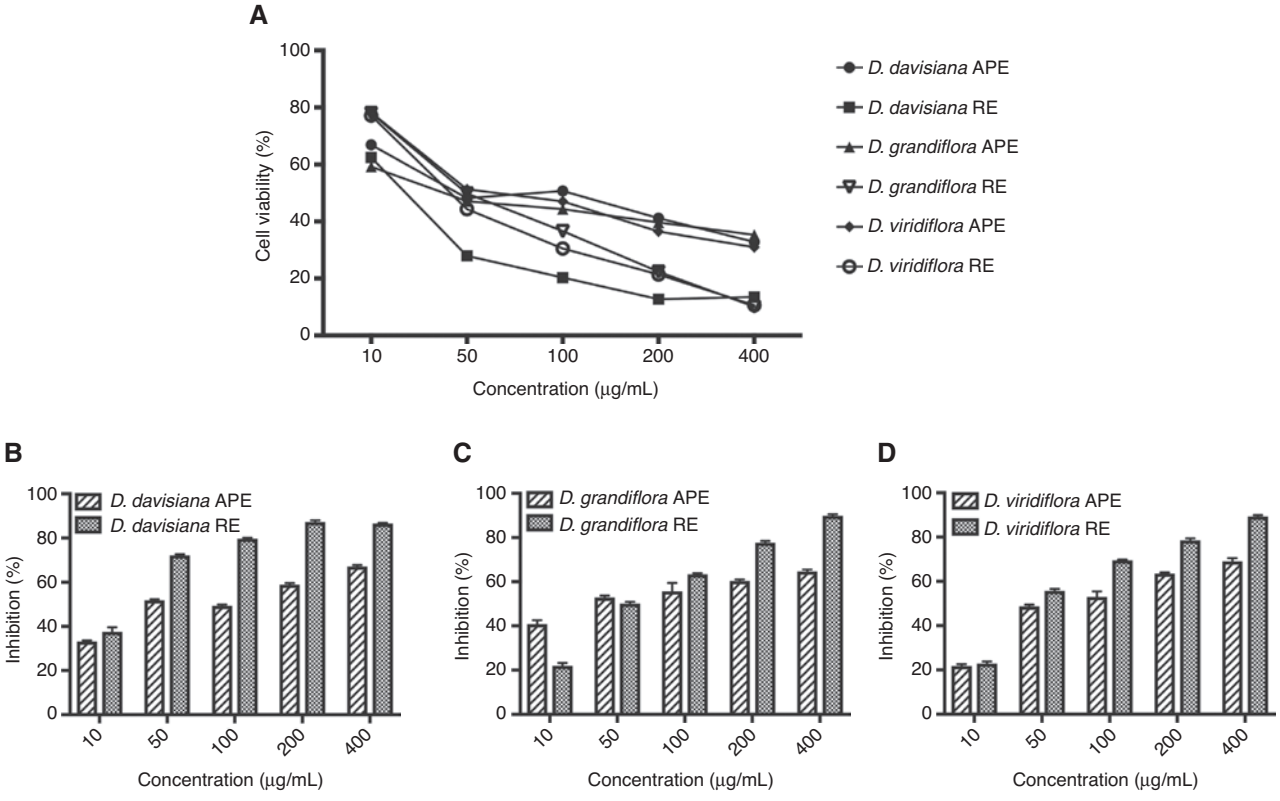


Figure 2: Screening cytotoxic activity of aqueous extracts obtained from the aerial parts and roots of *D. davisiana, D. grandiflora* and *D. viridiflora* on HEp-2 cancer cells using MTT method.
(A) Aerial parts and root extracts of *D. davisiana, D. grandiflora* and *D. viridiflora* were evaluated together. Results are given as cell viability (%). (B) Cytotoxic activities of *D. davisiana* extracts are given as inhibition (%). (C) Cytotoxic activities of *D. grandiflora* extracts are given as inhibition (%). (D) Cytotoxic activities of *D. viridiflora* extracts are given as inhibition (%). Three independent test results were considered, averages and standard deviations were calculated and shown in the figures.

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Table 1: IC_{50} values of *Digitalis* extracts against HEp-2, HepG2 and 3Y1 cell lines.

Extracts	IC_{50} values (μg/mL)		
	Cell lines		
	HEp-2	HepG2	3Y1
D. davisiana APE	50.8	211.4	94.7
D. davisiana RE	19.7	520.8	174.0
D. grandiflora APE	54.8	513.5	301.8
D. grandiflora RE	63.1	1078.6	87.9
D. viridiflora APE	79.6	1011.7	772.3
D. viridifora RE	51.3	2152.9	150.4

Cytotoxic activity of the extracts for HepG2 cells showed high IC_{50} values in a range of 211.4–2152.9 μg/mL (Figure 3, Table 1). The aerial parts were found to be more active than the roots against HepG2 cells (Figure 3). *Digitalis davisiana* APE showed the highest cytotoxicity among the tested extracts with a IC_{50} value of 211.4 μg/mL. All of the extracts showed concentration dependent low cytotoxicity against HepG2 cells (Figure 3), which suggests that these extracts showed a selective cytotoxicity against different cancer cells. This selectivity is important in the discovery of new anticancer agents.

Selectivity between cancer and non-cancerous cells is important for future drug development. Thus in this study, 3Y1 cells was chosen to determine the selectivity. Except *D. viridiflora* APE, all other extracts showed concentration dependent cytostatic activity (cell-growth inhibitory activity) on 3Y1 cell lines (Figure 4). Because, cytotoxic activity is not significant, even at 800 μg/mL (cell viability %, is in a range of 38–45). The difference between cytotoxic activity and cytostatic activity is that in cytostatic activity the decrease in cell number is due to the suppression of the cells without causing cell death, whereas in cytotoxic activity, cell death is the cause of

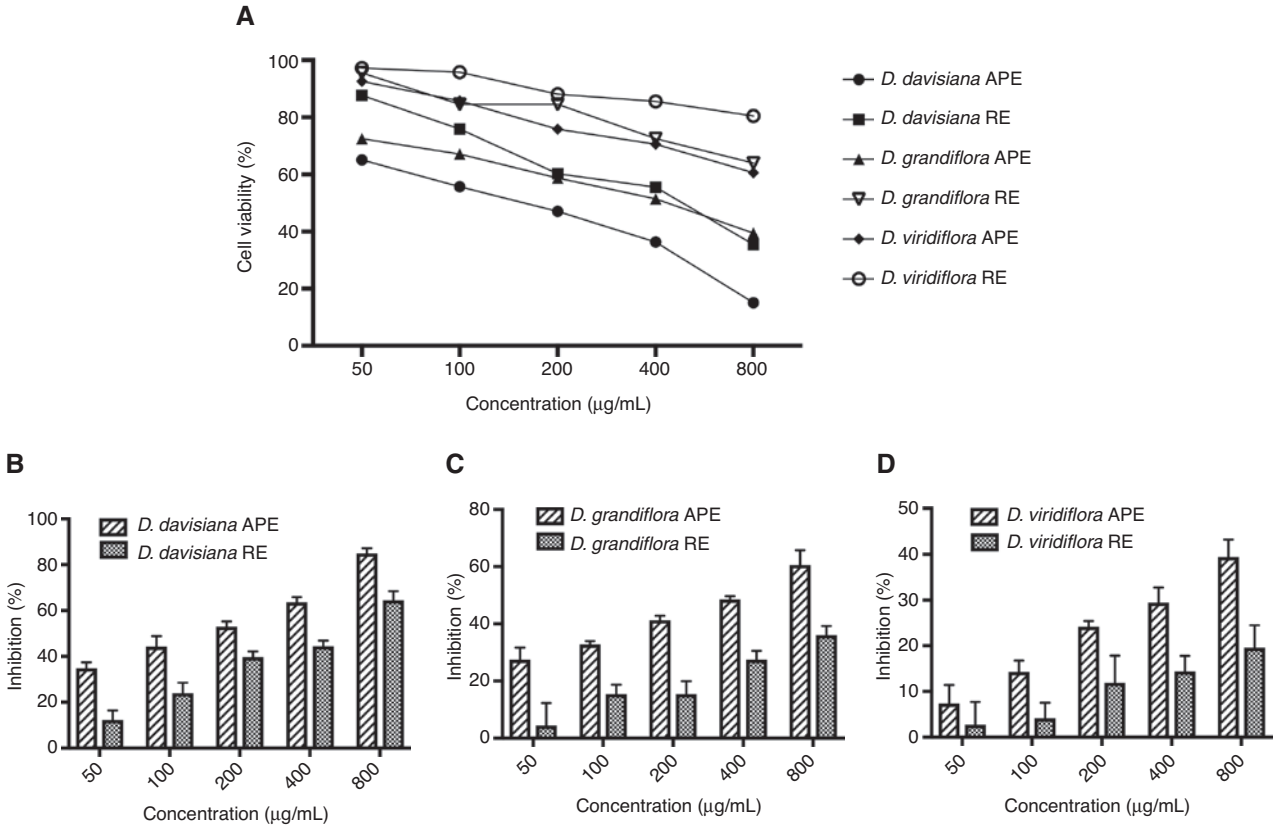


Figure 3: Screening cytotoxic activity of aqueous extracts obtained from the aerial parts and roots of *D. davisiana, D. grandiflora* and *D. viridiflora* on HepG2 cancer cells using MTT method.
(A) Aerial parts and root extracts of *D. davisiana, D. grandiflora* and *D. viridiflora* were evaluated together. Results are given as cell viability (%). (B) Cytotoxic activities of *D. davisiana* extracts are given as inhibition (%). (C) Cytotoxic activities of *D. grandiflora* extracts are given as inhibition (%). (D) Cytotoxic activities of *D. viridiflora* extracts are given as inhibition (%). Three independent test results were considered, averages and standard deviations were calculated and shown in the figures.

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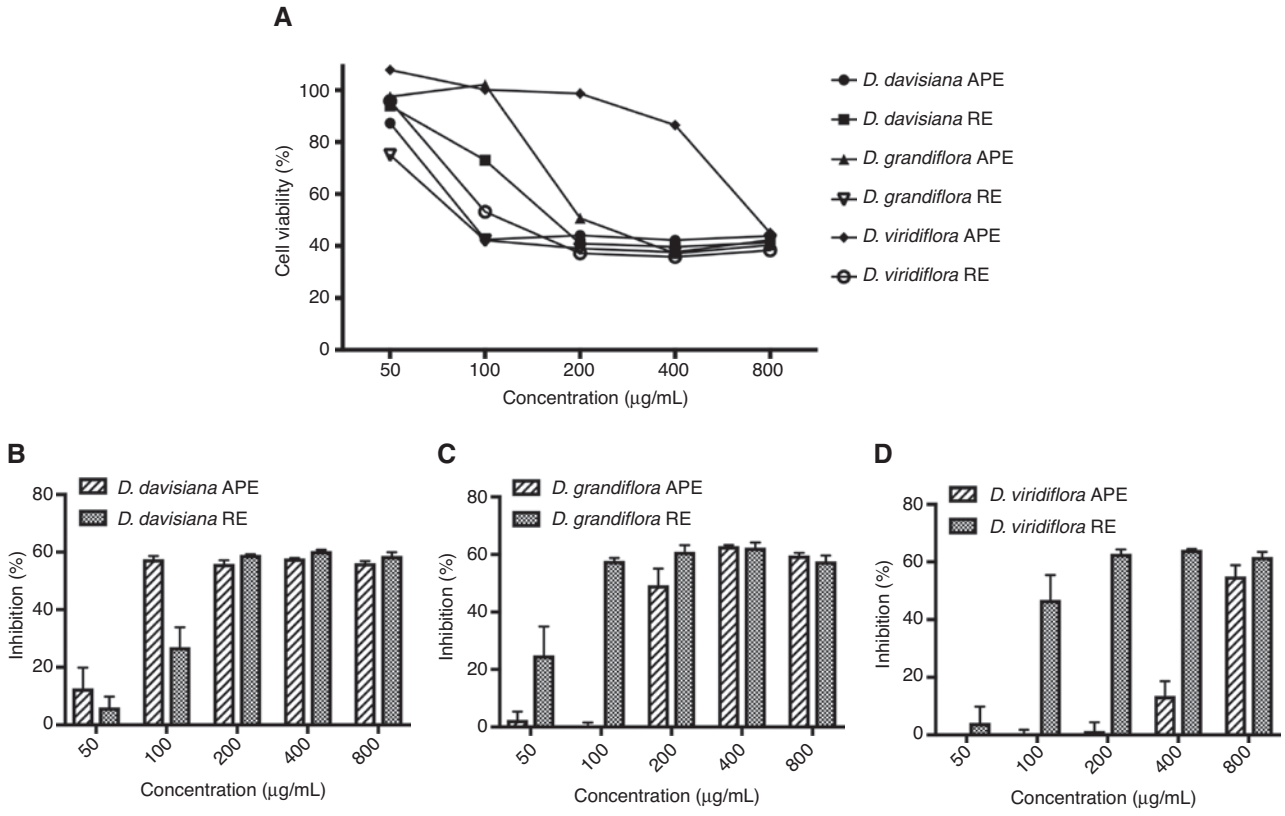


Figure 4: Screening cytotoxic activity of aqueous extracts obtained from the aerial parts and roots of *D. davisiana, D. grandiflora* and *D. viridiflora* on 3Y1 non-cancerous cells using MTT method.
(A) Aerial parts and root extracts of *D. davisiana, D. grandiflora* and *D. viridiflora* were evaluated together. Results are given as cell viability (%). (B) Cytotoxic activities of *D. davisiana* extracts are given as inhibition (%). (C) Cytotoxic activities of *D. grandiflora* extracts are given as inhibition (%). (D) Cytotoxic activities of *D. viridiflora* extracts are given as inhibition (%). Three independent test results were considered, averages and standard deviations were calculated and shown in the figures.

the decrease in cell number. *Digitalis davisiana* APE and *D. grandiflora* RE were found to be the most active extracts among tested extracts with 87.9 and 94.6 µg/mL IC_{50} values respectively, whereas *D. viridiflora* APE extract showed very low cytostatic activity against 3Y1 cells with 772.3 µg/mL IC_{50} value. Even at 400 µg/mL, there is no significant change in cell number: cell viability is more than 80% with the treatment of *D. viridiflora* APE (Figure 4).

Discussion

As a result, both aerial parts and roots of the selected plants showed similarly high potential of cytotoxic activity on HEp-2 cells. However, cytotoxicity on HepG2 cell line was found to be lower than that of HEp-2 cells. These findings indicated that different *Digitalis* extracts showed a selective cytotoxicity against HEp-2 cancer cells. IC_{50} values of the extracts against HEp-2 cells are found to be lower than that of HepG2 cells and 3Y1 cells (Table 1).

The previous studies on *Digitalis* species in cancer were focused mainly on the isolated compounds from the genus. Promising cytotoxic activity was reported for cardioactive glycosides and phenylethanoid glycosides already [6, 7, 20, 21]. In our previous study, cytotoxic activities of *Digitalis ferruginea* subsp *schischkinii* and *D. lamarckii* aerial parts extracts at 400 µg/mL concentration on HEp-2 cells were found to be 80% and 60% respectively [22]. Results of the present study have supported the previous studies. In our present study, at 400 µg/mL concentration, aerial parts of *D. davisiana*, *D. grandiflora* and *D. viridiflora*, showed 67.2%, 64.7% and 69% inhibition on HEp-2 cells, respectively (Figure 2).

Our findings support the claim that such plants, rich in cardenolides and phenolic compounds, may have been responsible for the cytotoxic activities of the tested *Digitalis* species.

In conclusion, *Digitalis* extracts have selective cytotoxic activity on both tested cancer and non-cancerous cell lines. Moreover, the selectivity was also seen between different cancer cells. This selectivity is important for

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the discovery of new anticancer agents. However, any relationships could not be found between cytotoxicity and LXRα agonist activity conducted by LXRE reporter gene assay, due to the low agonist effects of *Digitalis* extracts because of extreme dilutions. Further investigation is needed to clarify the mechanism of this activity.

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Conflict of interest statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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